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| <b>UTILITY PATENT APPLICATION TRANSMITTAL</b><br>(Only for new nonprovisional applications under 37 CFR § 1.53(b)) |  | <b>Attorney Docket No.</b> 0623.0820001/REF                                |
|  |  | <b>First Inventor or Application Identifier</b> John Edward HESKETH et al. |
|  |  | <b>Title</b> Regulation of Protein Secretion                               |
|  |  | <b>Express Mail Label No.</b>  |

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| <b>APPLICATION ELEMENTS</b><br>See MPEP chapter 600 concerning utility patent application contents   |  | <b>ADDRESS TO:</b><br>Assistant Commissioner for Patents<br>Box Patent Application<br>Washington, DC 20231  |  |
| 1. <input type="checkbox"/> <b>* Fee Transmittal Form (e.g., PTO/SB/17)</b><br>(Submit an original, and a duplicate for fee processing)  |  | 6. <input type="checkbox"/> <b>Microfiche Computer Program (Appendix)</b>   |  |
| 2. <input checked="" type="checkbox"/> <b>Specification</b> [Total Pages <u>28</u> ]<br>(preferred arrangement set forth below)<br>• Descriptive title of the Invention<br>• Cross References to Related Applications<br>• Statement Regarding Fee sponsored R & D<br>• Reference to Microfiche Appendix<br>• Background of the Invention<br>• Brief Summary of the Invention<br>• Brief Description of the Drawings (if filed)<br>• Detailed Description<br>• Claim(s)<br>• Abstract of the Disclosure  |  | 7. <b>Nucleotide and/or Amino Acid Sequence Submission (if applicable, all necessary)</b><br>a. <input type="checkbox"/> <b>Computer Readable Copy</b><br>b. <input type="checkbox"/> <b>Paper Copy (identical to computer copy)</b><br>c. <input type="checkbox"/> <b>Statement verifying identity of above copies</b>   |  |
| 3. <input checked="" type="checkbox"/> <b>Drawing(s) (35 U.S.C. 113)</b> [Total Sheets <u>4</u> ]<br>4. <input type="checkbox"/> <b>Oath or Declaration</b> [Total Pages _____]<br>a. <input type="checkbox"/> <b>Newly executed (original or copy)</b><br>b. <input type="checkbox"/> <b>Copy from a prior application (37 CFR 1.63(d)) (for continuation/divisional with Box 17 completed)</b><br>[Note Box 5 below]<br>i. <input type="checkbox"/> <b>DELETION OF INVENTOR(S)</b><br>Signed statement attached deleting inventor(s) named in the prior application, see 37 CFR §§ 1.63(d)(2) and 1.33(b). |  | <b>ACCOMPANYING APPLICATION PARTS</b><br>8. <input type="checkbox"/> <b>Assignment Papers (cover sheet &amp; document(s))</b><br>9. <input type="checkbox"/> <b>37 CFR 3.73(b) Statement</b> <input type="checkbox"/> <b>Power of Attorney</b><br>(when there is an assignee)<br>10. <input type="checkbox"/> <b>English Translation Document (if applicable)</b><br>11. <input type="checkbox"/> <b>Information Disclosure Statement (IDS)/PTO-1449</b> <input type="checkbox"/> <b>Copies of IDS Citations</b><br>12. <input type="checkbox"/> <b>Preliminary Amendment</b><br>13. <input checked="" type="checkbox"/> <b>Return Receipt Postcard (MPEP 503)</b><br>(Should be specifically itemized)<br>14. <input type="checkbox"/> <b>*Small Entity Statement(s)</b> <input type="checkbox"/> <b>Statement filed in prior application, Status still proper and desired</b><br>15. <input type="checkbox"/> <b>Certified Copy of Priority Document(s)</b><br>(if foreign priority is claimed)<br>16. <input type="checkbox"/> <b>Other: 37 C.F.R. § 1.136(a)(3) Authorization</b><br><input type="checkbox"/> <b>Other:</b><br>*NOTE FOR ITEMS 14 & 15: IN ORDER TO BE ENTITLED TO PAY SMALL ENTITY FEES, A SMALL ENTITY STATEMENT IS REQUIRED (37 C.F.R. § 1.27), EXCEPT IF ONE FILED IN A PRIOR APPLICATION IS RELIED UPON (37 C.F.R. § 1.28) |  |
| 5. <input type="checkbox"/> <b>Incorporation By Reference (useable if Box 4b is checked)</b><br>The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.  |  |   |  |
| 17. If a CONTINUING APPLICATION, check appropriate box, and supply the requisite information below and in a preliminary amendment.<br><input checked="" type="checkbox"/> <b>Continuation</b> <input type="checkbox"/> <b>Divisional</b> <input type="checkbox"/> <b>Continuation-in-Part (CIP)</b> of prior international appl. No: PCT/GB98/02664<br>Prior application information: Examiner _____ Group/Art Unit: _____   |  |   |  |

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| SIGNATURE         |                  | Date                              | March 2, 2000 |

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**Box Patent Application**

Re: U.S. Non-Provisional Continuation Utility Patent Application Under  
37 C.F.R. § 1.53(b)  
Based on Int'l. Appl. No. PCT/GB98/02664; Int'l. Filing Date: September 4, 1998  
Appl. No. To Be Assigned; Filed: HERewith (March 2, 2000)  
For: **Regulation of Protein Secretion**  
Inventors: John Edward HESKETH; Albert TAULER; Ian Fraser PRYME  
Our Ref: 0623.0820001/REF

Sir:

The following documents are forwarded herewith for appropriate action by the U.S.  
Patent and Trademark Office:

1. U.S. Utility Patent Application entitled:

**Regulation of Protein Secretion**

and naming as inventors:

John Edward HESKETH;  
Albert TAULER; and  
Ian Fraser PRYME

and claiming priority to GB Application No. 9718908.8, filed September 5, 1997;

the application comprising a total of 28 pages:

1c490 U.S. PTO  
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Assistant Commissioner for Patents

March 2, 2000

Page 2

a. A specification containing:

(i) 25 pages of description prior to the claims;

(ii) 2 pages of claims (8 claims);

(iii) a one (1) page abstract;

b. 4 sheets of drawings: (Figures 1-5, 6A and 6B);

2. USPTO Utility Patent Application Transmittal Form PTO/SB/05;
3. 37 C.F.R. § 1.136(a)(3) Authorization to Treat a Reply As Incorporating An Extension of Time (in duplicate); and
4. Two (2) return postcards.

It is respectfully requested that, of the two attached postcards, one be stamped with the filing date of these documents and returned to our courier, and the other, prepaid postcard, be stamped with the filing date and unofficial application number and returned as soon as possible.

This patent application is being submitted under 37 C.F.R. § 1.53(b) without Declaration and without filing fee.

Respectfully submitted,

STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.



Raz E. Fleshner  
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REF/neh  
Enclosures

## REGULATION OF PROTEIN SECRETION

The present invention relates to the secretion of proteins from mammalian cells.

- 5 Mammalian cell lines are increasingly being used to produce commercially significant amounts of recombinant proteins in cell factories and in transgenic animals. There are numerous examples where normally secreted proteins are being produced in this way (Bock *et al* 1982, Michel *et al* 1985, Lin *et al* 1986, Devlin *et al* 1987, Shak *et al* 1990), as well as a number of commercial vectors for the
- 10 secretion of heterologous proteins. However such systems have been largely used for the production of secreted proteins rather than proteins which are normally intracellular and secretion of intracellular proteins in mammalian expression systems is an increasingly important aim in biotechnological research (James and Simpson, 1996). The present inventors are only aware of one paper describing the successful
- 15 secretion of an intracellular protein from mammalian cells (Lee *et al*, 1996). This protein (sialyltransferase), however, is normally synthesised in the endoplasmic reticulum (ER) and successful secretion did not depend on an initial redirection of the mRNA.
- 20 It is well established that specific mRNAs are translated in specific subcellular locations, for instance membrane and secreted proteins are translated on the rough endoplasmic reticulum in association with membrane-bound polysomes (Blobel and Dobberstein, 1975), and the mRNAs for proteins such as c-myc (Hesketh *et al*, 1994, metallothionein I (Mahon *et al* 1995), ribosomal proteins and cyclin A
- 25 (Hovland *et al*, 1995) are translated in association with cytoskeletal-bound polysomes (for reviews see Hesketh 1996, Hovland *et al* 1996). It now appears that the protein synthetic apparatus is compartmentalised into at least three polysome populations - free (FP), cytoskeletal-bound (CBP) and membrane-bound (MBP) polysomes. These different populations of mRNAs can be separated using a

sequential detergent/salt extraction (Vedeler *et al* 1991). Different signals direct these mRNAs to their site for translation. In the case of membrane and secreted proteins it has been well established that the signal peptide is required to target the ribosome-mRNA complex to the endoplasmic reticulum whilst recent evidence has  
5 shown that for mRNAs localised in the cytoplasm or associated with the cytoskeleton there are signals in the 3'untranslated region (Kislauskis *et al* 1994, Hesketh *et al* 1994, Wilson *et al* 1995, Veyrune *et al* 1996).

According to the present invention there is provided an RNA molecule encoding a  
10 mammalian signal peptide operatively linked to a protein that would normally not be secreted from a mammalian cell, said signal peptide allowing at least some of said protein to be synthesised on the endoplasmic reticulum in a manner so that it can be secreted from said mammalian cell.

15 Without being bound by theory, it is believed that the signal peptide is provided initially and then translation of the mRNA stops or slows down to allow the signal peptide to bind to a signal recognition particle and direct ribosomes associated with the mRNA to the endoplasmic reticulum. Translation then starts again/speeds up to allow the rest of the protein to be translated.

20 The term "protein" is used herein in a broad sense to include moieties comprising amino-acids linked by peptide bonds. It therefore includes peptides and polypeptides.

25 The present invention is widely applicable. In principle the sequence of any naturally occurring mammalian signal peptide that is normally associated with a protein secreted from mammalian cells can be used to design appropriate RNA molecules encoding a mammalian signal peptide for use in the present invention.

Variants of such naturally occurring signal peptides that can be used to secrete such a protein may also be used and, for the purposes of the present invention, are deemed to be within the scope of the term "mammalian signal peptide ". Preferred such variants have at least at least 50% amino acid sequence identity with naturally occurring mammalian signal peptide sequences. More preferably the degree of sequence identity is at least 75%. Sequence identities of at least 90% or of at least 95% are most preferred. For the purposes of the present invention sequence identity can be determined by using the "BESTFIT" program of the Wisconsin Sequence Analysis Package GCG 8.0.

Preferred signal peptide sequences include: rat albumin (Database Accession Number J00698), bovine growth hormone, lactalbumin and other milk proteins.

A skilled person is able to ascertain the signal sequences of many proteins from available database sequences or publications. Even if these sequences are not published, for example, sequence homology studies may be used to compare a candidate amino-acid sequence with amino acid sequences of known signal sequences.

In a preferred embodiment of the present invention the RNA molecule does not include a sequence that might direct the molecule to an intracellular location other than the endoplasmic reticulum or to free and/or to cytoskeletal bound polysomes, or it includes such a sequence but in a form that is modified to reduce its effectivity in directing molecules to an intracellular location other than the endoplasmic reticulum or to free and/or to cytoskeletal bound polysomes (relative to the corresponding naturally occurring sequence).

In this preferred embodiment of the present invention possible competition between a signal sequence directing a protein to the endoplasmic reticulum and an RNA sequence directing RNA encoding the protein to an intracellular location other than the endoplasmic reticulum or to free and/or to cytoskeletal bound polysomes can be avoided, or at least altered so as to increase the probability of proteins being directed to the endoplasmic reticulum.

This can be achieved by providing the RNA molecule with a deletion, insertion or substitution in respect of all or part of a 3' untranslated (UTR) region relative to the corresponding region present in naturally occurring RNA encoding the protein that would normally not be secreted from a mammalian cell. Signals present in 3'UTR regions of RNA molecules directed to specific subcellular locations or to free or cytoskeletal - bound polysomes are believed to control the localisation of such mRNAs, at least to some degree and mutations in such regions can inactivate the signals. The signals in the 3' UTR regions of RNA molecules can be identified by deletion or mutation of sequences within the 3'UTR region of said mRNA molecules and assay of localisation capabilities by linking to a reporter gene and transfection into mammalian cells in culture followed by hybridisation assays to detect mRNA distribution.

DNA molecules capable of being transcribed to provide the RNA molecules discussed above are also within the scope of the present invention. They may be provided in the form of a vector (e.g. a plasmid, phage or cosmid), although this is not essential.

Also within the scope of the present invention is a mammalian cell comprising a DNA or RNA molecule as described above. The mammalian cell may be present in cell culture or in a non-human animal. The term "cell culture" is used herein to mean one or more cells that are not present in an animal and that can be used to

express proteins under appropriate conditions. Usually such cells will be maintained in an appropriate growth medium under buffered conditions. The cell culture may be e.g. a culture of mammalian fibroblasts, a CHO, BHK or myeloma cell culture. The non-human animal may be a transgenic animal. (The term "transgenic animal" includes transkaryotic animals.) It may secrete heterologous proteins in its milk.

The present invention also includes a method obtaining a protein from a mammalian cell comprising the step of expressing the protein using a nucleic acid molecule of the present invention and allowing the cell to secrete the protein. The protein may then be purified by known purification techniques.

A chimaeric protein comprising a mammalian signal sequence linked to a protein that would normally not be secreted from a mammalian cell is another aspect of the present invention. Such a protein can be produced by translating an RNA molecule of the present invention.

In addition to the other aspects of the present invention, it should be noted that the present invention provides nucleic acid molecules that hybridise to the nucleic acid molecules discussed above. These may be useful e.g. as probes, as primers, or as antisense molecules for use in altering expression, etc.

Desirably such hybridising nucleic acid molecules are at least 10 nucleotides in length and preferably are at least 25 or at least 50 nucleotides in length. They may hybridise under stringent hybridisation conditions. One example of stringent hybridisation conditions is where attempted hybridisation is carried out at a temperature of from about 35°C to about 65°C using a salt solution which is about 0.9 molar. However, the skilled person will be able to vary such parameters as



appropriate in order to take into account variables such as probe length, base composition, type of ions present, etc.

- 5 Although the present invention has been described above largely with reference to naturally occurring mammalian signal peptides or variants thereof, in its broadest scope all signal peptides capable of functioning in mammalian cells may be used. These may be naturally or non-naturally occurring. Preferably (but not essentially) they do not occur naturally in prokaryotic organisms (e.g. *E. Coli*).

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The present invention will now be described by way of example only with reference to the accompanying figures, wherein

- 5      **Figure 1**      shows a schematic diagram of the constructs described in the Material and Methods. The parent vector is pcDNA3. The solid square represents the native 5'UTR of rabbit beta-globin. SS is the rat albumin signal sequence.
- 10      **Figure 2**      shows a Northern Hybridisation of total RNA from polysomes isolated from transfected cells. All lanes were loaded with 10  $\mu$ g RNA; the filter was hybridised with a rabbit beta-globin cDNA probe and specific bands detected by autoradiography at  $-70^{\circ}\text{C}$  for 3 days using Hyperfilm-MP. F, free polysomes; C, cytoskeletal-bound polysomes; M, membrane-bound polysomes.
- 15      **Figure 3**      shows a quantification of the distribution of globin mRNA in transfected cells. Results are expressed in arbitrary units mRNA per unit 18S rRNA obtained from direct radioactivity imaging using a Canberra Packard Instantimager. Abundance values were normalised taking the abundance in free polysomes as 100 for each experiment. Results are means  $\pm$  S.E.M (n=4). Groups were compared using Students 't' test: transcript abundance in the membrane-bound polysomes was significantly different between GG and SSGG cells ( $p=0.01$ ), GM and SSGM ( $p<0.05$ ) using a two-tailed test and between SSGM and SSGG using a one-tailed test.
- 20
- 25      **Figure 4**      shows the mRNA stability of the transcripts. Cells were grown to 70% confluence and transcription inhibited with Actinomycin D. RNA was extracted at time points then analysed by Northern Blotting.

Quantification was carried out by direct radioactivity imaging using a Canberra Packard Instantimager. Results are means  $\pm$  S.E.M and are expressed as a percentage of initial abundance.

5 GG and SSG (n=6)

10 **Figure 5** shows polysome profiles of transfected Ltk<sup>-</sup> cells. Soluble detergent/salt extracted material was layered on to a 15%-40% sucrose gradient and centrifuged at 200 000  $\times$  g for 1 hour. Polysome profiles were monitored by measuring the absorbance at 260 nm using a flow cell.

**Figure 6** shows a Northern Hybridisation and quantification of SSGA.

15 **Figure 6A:** Northern Hybridisation of Ltk<sup>-</sup> cells transfected with pcKSSGA. All lanes were loaded with 20  $\mu$ g RNA. The filter was hybridised with a rabbit beta-globin cDNA probe.

**Figure 6B:** Quantification of mRNA abundance.

20 Results are expressed in arbitrary units of mRNA abundance per unit 18S rRNA obtained from direct radioactivity imaging and normalised by taking the abundance in free polysomes at 100 for each experiment. Results are means  $\pm$  S.E.M. (n=3).

25

## EXAMPLES

The approach taken involved three stages: to construct a series of vectors with the albumin signal peptide and different 3'untranslated region (3'UTR) sequences linked to the rabbit beta-globin 5' untranslated region and coding sequence as a reporter gene; to introduce these constructs into cells by stable transfection and then to determine the subcellular location of the mRNA, and its stability and translational efficiency.

### Materials and Methods

**Bacteria and cell culture:** Plasmids were cloned and propagated in a standard manner. CHO K1 cells were grown in Ham's F12 medium and Ltk<sup>-</sup> cells, a mouse fibroblast line, were grown in Dulbecco's minimal Eagle's medium. Media were supplemented with 10% foetal calf serum and grown in an atmosphere of 5% CO<sub>2</sub>.

Cells were propagated in 90mm Petri dishes for fractionation and in 35mm Petri dishes for transfection.

**Vector construction:** All constructs (Figure 1) were derived from rabbit  $\beta$ -globin coding sequences by PCR cloning with the primers described in Table 1 and all contained the native globin 5' UTR. Each construct was verified by sequencing using an ABI 373A sequencer and cycle sequencing. The construction of pcKGG, a plasmid expressing rabbit beta-globin with native 5' and 3' UTRs has been described previously (Mahon *et al*, 1997). pcKGM expresses globin with the 3' UTR of mouse c-myc and was made as follows. A portion of the globin coding region and the entire c-myc 3'UTR was amplified by PCR using primers KP4 and KP5, KP4 maintaining a BamHI site in the globin coding region and KP5 creating an XbaI site downstream of the c-myc poly-A signal, and plasmid MP13 as template (Veyrune et al 1996). Using 1 ng template and 20 pmol each primer in 1.5 mM

MgCl<sub>2</sub> and 4 Units Taq Polymerase (PerkinElmer), the reaction was cycled at 94 C for 1 min, 55 C for 1 min, and 72 C for 2 mins. The fragment and pcKGG were digested with BamHI and KpnI and ligated using standard methods (Sambrook et al 1989).

5

Making the signal sequence constructs required creation of an appropriate restriction site at the AUG codon. This was accomplished by PCR using primers KP6 and KP7; KP6 matching the sequence of pcDNA3 at position 731 and KP7 creating an NdeI site while maintaining the AUG and reading frame. The template was pcKGG, the reaction was with the conditions described above. The product of the reaction was purified, then 5 µg was used in a PCR megaprime with primer KP8 (covering a sequence in the globin coding region which includes a BamHI site), 1 µg pcKGG as the template and the cycles as described above. The resulting fragment was subcloned into pBluescript in the KpnI and BamHI sites, creating pBS-Nde.

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The signal sequence was provided by annealing synthetic oligodeoxynucleotides that corresponded to the rat albumin signal sequence (SS1 and SS2). The insert was ligated into the NdeI site of pBS-Nde, creating pBS-SS. The KpnI-BamHI fragment from pBS-SS covering the 5' UTR, albumin signal sequence and globin coding region to the BamHI site, was then ligated into appropriately digested pcKGG and pcKGM to create pcKSSGG and pcKSSGM.

20

25

The albumin 3'UTR was removed from the rat albumin gene by restriction and after treatment of the restricted globin vector with Vent polymerase the albumin 3'UTR was introduced by blunt-ended ligation. pcKSSGA was constructed as described for pcKSSGG and pcKSSGM.

**Transfection:** Transfections were carried out with LipofectAMINE™ (Gibco) according to the manufacturer's instructions. Cells were subcultured on to 35 mm

Petri dishes at a density of  $5 \times 10^5$  cells/dish and grown to 70-80% confluence. The cells were overlaid with 1  $\mu$ g of plasmid DNA and 6-10  $\mu$ l LipofectAMINE in serum-free medium. After 5 hours, serum was added to 10% final concentration. At 24 hours the medium was replaced with complete medium and selection with 200  $\mu$ g/ml G418 was begun at 72 hours. Cells were maintained in 100  $\mu$ g/ml G418 once stably transfected. The cell lines are named for the transfected construct, e.g. the cells transfected with pcKGG is called GG, etc.

**Cell Fractionation:** Ltk- cells were grown to 70% confluence, harvested using a rubber policeman and fractionated by a sequential detergent/salt extraction procedure (Vedeler et al,1991; Hesketh et al 1994). Polysomes were separated from monosomes and lighter ribonucleoprotein particles by centrifugation at 32,000 g for 17 hours through a 15 ml cushion of 40% sucrose (Hovland et al,1995).

**RNA Extraction and RNA Gel Electrophoresis:** Total RNA was extracted by the acid/guanidinium/phenol/chloroform method of Chomczynski and Sacchi (1987), and the preparations were assessed by the A260/A280 . RNA was then separated by electrophoresis through a denaturing 2.2 M formaldehyde, 1.2% agarose gel (Sambrook et al 1989) and transferred to a nylon membrane (Genescreen, NEN Dupont, Ltd) by capillary blotting. RNA was fixed to the membrane by UV light and stored dry.

**Northern Hybridisation and DNA Probes:** Membranes were pre-hybridised at 42 C for a minimum of 6 hours with 0.1 mg/ml denatured salmon sperm DNA in 50% formamide, 10% dextran sulphate, 0.2% bovine serum albumin, 0.2% polyvinylpyrrolidone, 0.2% Ficoll, 0.1% sodium pyrophosphate, 1% SDS and 50 mM Tris HCl, pH 7.5. The beta-globin cDNA probe was described previously (Veyrune et al 1996). The c-myc probe was a cDNA of the 3 exons of the murine gene (Hesketh et al,1994) and was the gift of Dr. M. Cole (Princeton University,

New Jersey USA), the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was a 0.78kb *PstI-XbaI* fragment from the human cDNA (American Tissue Culture Collection, accession number 57090) and the 18S rRNA probe was a 1.4kb *BamHI* fragment from the cDNA obtained from Dr. R. Fulton, Beatson Institute, Glasgow.

- 5 20-30 ng of probe was labelled with  $^{32}\text{P}$  dCTP by random priming (Megaprime kit from Amersham International UK). The labelled probe was added to the pre-hybridisation mix and hybridised overnight at 42°C. The filters were washed briefly at room temperature in 2XSSC to remove hybridisation mix. Washes at 65°C were probe specific as follows: globin-1X SSC 0.5% SDS 2X 30 min., c-myc-10 0.5X SSC, 1% SDS 2X 1 hr., GAPDH-1X SSC, 1% SDS 2X 1 hr., 18S-0.2X SSC, 1% SDS. A final brief wash in 0.1X SSC removed SDS. Specific hybridisation was detected and quantified on a Canberra Packard Instantimager. The amounts of specifically bound probe was corrected for non-specific binding and the data expressed per unit of rRNA as measured by hybridisation to the 18S rRNA probe.
- 15 Before being reprobed filters were stripped by heating them to 95°C in 0.1% SDS for 10 min.

- mRNA stability: mRNA stability was determined by measuring abundance over a 2-12h time period following the inhibition of transcription. Cells were grown to 20 70% confluence and transcription was inhibited by the addition of Actinomycin D (5 µg/ml). RNA was extracted as described above.

- Polysome profiles and Translational Efficiency: Cells were grown to 70% confluency, scraped into the medium using a rubber policeman, then pelleted at 25 2000 rpm for 5 min. at 4°C. Cells were washed in DEPC PBS, and pelleted as before. The cells were resuspended in a solution of 10 mM TEA (triethanolamine) pH 7.6, 130 mM KCL, 5 mM MgCl<sub>2</sub>, 0.5 mM CaCl<sub>2</sub>, 0.25 mM sucrose with 0.5% deoxycholate and 0.5% Triton X100 incubated on ice 10 minutes. The nuclei were

5 pelleted by centrifuging at 3000 rpm for 10 min at 4 C. 0.3 ml of the supernatant  
 were loaded onto a 7 ml 15%-40% sucrose gradient and centrifuged at 200,000 x g  
 for 1 hour and then monitored by measuring the absorbance at 260 nm using a flow-  
 through cell mounted in a Zeiss PM 2A spectrophotometer and coupled to a W + W  
 recorder. The outflow from the flow cell was collected and each gradient split into  
 three fractions - polysomes, monosomes, and remainder (untranslated material).  
 Polysomes were pelleted by centrifuging at 32000 x g for 17 hours and RNA was  
 extracted from the pellet. Monosomal RNA was precipitated with isopropanol and  
 ammonium acetate, then extracted as described earlier.

## 10 Results

15 **Redirection of globin mRNA by albumin signal sequence:** Initially, four plasmids  
 were constructed based on the rabbit beta-globin 5'UTR and coding region together  
 with either the native 3'UTR or the c-myc 3'UTR and both with and without the  
 albumin signal sequence. These constructs were introduced into Ltk- fibroblasts  
 and stable transfected cell lines were established. Expression of the transfected gene  
 was assessed by Northern hybridisation to detect globin transcripts and all four  
 genes were found to be expressed. We found the highest levels of globin expression  
 at 70% confluence (data not shown) and all subsequent experiments were carried out  
 20 at that stage of growth.

25 Detergent/salt fractionation was carried out to investigate the compartmentation of  
 the transcripts between FP,CBP and MBP. Each cell line was fractionated,  
 polysomes pelleted, and then RNA extracted from the pellets and analysed by  
 Northern hybridisation for abundance of globin transcripts. As shown in figure 2,  
 in the control cell lines (GG) the globin transcript was found largely in free  
 polysomes. In contrast, in cells expressing the globin transcript with the albumin  
 signal sequence (SSGG) this transcript was found predominantly in the MBP/ER.  
 The SSGM transcript was also found at highest abundance in the MBP/ER fraction.



Reprobing of filters with the 18S rRNA probe allowed correction for RNA loading and quantification of the hybridisation data per unit RNA. The abundance of globin transcripts in FP, CB and MBP was respectively  $2.5 \pm 0.4$ ,  $2.9 \pm 1.0$  and  $2.1 \pm 0.9$  (means from at least four separate experiments  $\pm$  s.e.m., expressed as arbitrary units of c.p.m. globin probe bound/c.p.m. 18S probe bound) in GG cells and  $0.9 \pm 0.2$ ,  $1.3 \pm 0.2$  and  $2.5 \pm 0.8$  in SSGG cells. Since it was the relative transcript distribution rather than overall expression level that was the most important parameter in these experiments, these data were then expressed relative to the abundance in FP fraction; as shown in Figure 3 these data confirm that the addition of albumin signal sequence redirects the globin mRNA to the ER ( $p = 0.01$ ).

In the GM cells the abundance of globin transcripts in FP, CBP and MBP was respectively  $2.6 \pm 0.7$ ,  $2.8 \pm 0.9$  and  $1.6 \pm 0.5$  (means from at least four separate experiments  $\pm$  s.e.m., expressed as arbitrary units of c.p.m. globin probe bound) and in SSGM cells the abundance distribution was respectively  $2.5 \pm 1.1$ ,  $2.3 \pm 0.8$  and  $3.0 \pm 1.4$ . Analysis of these data expressed as abundances relative to that in FP showed that the GM transcripts were present largely in CBP, as found previously in (Hesketh *et al.*, 1994) but that, in contrast, the SSGM transcripts were found at similar relative abundances in CBP and MBP (Figure 3). The abundance of globin transcripts in MBP was significantly greater in SSGM cells than in GM cells, again showing the ability of the signal sequence to redirect transcripts to the ER. However, the increased abundance in MBP in SSGM cells compared to GM cells was significantly less ( $p < 0.05$ ) than the increased abundance in SSGG cells compared to GG cells. Thus, although the signal sequence redirected the globin coding region and 5'UTR with the c-myc 3'UTR attached, the presence of both c-myc 3'UTR and the signal sequence reduced the extent to which redirection occurred. This suggests that there is competition between an ER localising signal (signal peptide) and a cytoskeletal localising signal (c-myc 3'UTR; Veyrune *et al.*, 1996).

**Addition of the signal sequence has no effect on mRNA stability or translational efficiency:** Since it was theoretically possible that the manipulation of 5' signals may have altered the stability of the globin mRNA, we therefore determined the stability of the chimaeric transcripts. Each cell line was grown to 70% confluence and then treated with Actinomycin D to inhibit further transcription; RNA abundance was measured by Northern blotting. As globin is a highly stable message (Aviv *et al.*, 1976) the abundances of GG and SSGG transcripts were measured over a 12 h period. As shown in Figure 4, the addition of the albumin signal sequence did not significantly alter the stability of the globin mRNA. Similarly, analysis of GM SSGM cell lines showed that addition of the signal sequence to globin transcripts with the c-myc 3'UTR did not destabilise the mRNA (data not shown). Reprobing of the filters to detect GAPDH mRNA indicated that transcription had been inhibited in all four cell lines (data not shown).

**Effects of signal sequence on mRNA stability:** Since it was theoretically possible that the manipulation of 5' and 3' signals may have altered the stability of the globin mRNA, we therefore determined the stability effect of the added signal sequence on mRNA stability. Cells were grown to 70% confluence and then treated with Actinomycin D to inhibit further transcription; RNA abundance was measured by Northern blotting. As globin is a highly stable message (Aviv *et al.*, 1976) the abundance of GG and SSGG transcripts were measured over a 12 h period. As shown in Figure 4, the addition of the albumin signal sequence did not significantly alter the stability of the globin mRNA. Reprobing of the filters to detect GAPDH mRNA (data not shown) indicated that transcription had been inhibited in both cell lines.

**Translational efficiency:** In order to determine whether the manipulation of signals within the foreign gene had affected either translation in general or translation of the modified gene in particular, the protein synthetic apparatus of the four cell lines was

subjected to polysome profile analysis. The cells were lysed with salt and detergent, and soluble material loaded onto a 15%-40% sucrose gradient. Polysome profiles were recorded and are presented in Figure 5, and polysome/monosome ratios in Table 2. Polysome profiles were very similar for all four lines with a similar proportion of ribosomes present in active polysomes. The different peak heights represent differences in the total amount of RNA loaded onto the gradient. Thus, transfection with the 4 constructs did not affect overall protein synthesis. Analysis of the proportion of globin transcripts in polysome and monosome fractions from the sucrose gradients (Table 3) shows that addition of the signal sequence did not significantly alter the translation of the transcripts.

**The influence of the albumin 3'UTR:** The results in Figure 3 suggested that there is competition between 5' and 3' targeting signals but we were concerned that the observed effects were due to some form of interaction between the globin coding sequences and the c-myc 3'UTR which prevented the signal sequence performing its signalling role. To address this question we constructed a further vector with a different 3'UTR, namely the 3'UTR from the albumin gene: globin coding region with both the 3'UTR of rat albumin mRNA and the rat albumin signal sequence (SSGA). The plasmid was transfected into Ltk- cells and stable cell lines established. The SSGA cells were fractionated and analysed as described above. As shown in Figure 6 the results show that the SSGA transcripts containing both albumin 3'UTR and signal sequence were recovered predominantly in the MBP/endoplasmic reticulum fraction. This indicates that introduction of a foreign 3'UTR is not sufficient, *per se*, to prevent redirection of the globin transcript to the ER. A further vector (GA) was made in which the globin coding sequence was linked to the rat albumin 3'UTR but without the signal sequence; unfortunately, further analysis was not possible because we were unable to detect transfected cells expressing this gene.

### Discussion

The present results show that by a combination of DNA recombinant technology and transfection it is possible to produce cell lines in which the globin mRNA is retargeted to the endoplasmic reticulum. Thus, in Ltk- fibroblasts, the addition of the rat albumin signal sequence to globin with the native globin 3'UTR redirected the mRNA to membrane-bound polysomes. This is the first demonstration of redirection of a mRNA from free polysomes to the ER in a stable cell line. Partial redirection of histone mRNA to MBP has previously been demonstrated in mammalian cells (HeLa) using an *E. coli* signal sequence (Zambetti et al 1987).

Also, Lee and co-workers (1996) have secreted active sialyltransferase from COS-7 cells using an IgM signal peptide but in this case the native sialyltransferase is synthesised in the ER. Importantly, in each of these cases, however, transfections were transient.

It is now clear that, in addition to mRNAs translated on free polysomes, a considerable proportion of mRNAs are translated in polysomes associated with the cytoskeleton (Hesketh, 1996). The targeting of mRNAs to the cytoskeleton has been shown to be due to signals in the 3'UTR (Hesketh et al, 1994; Veyrune et al, 1996; Mahon et al, 1997). As shown in figure 3, if in addition to a signal peptide, a cytoskeletal targeting signal is present in the 3'UTR of the transcript the shift to membrane-bound polysomes is decreased. This appears not to be caused by a non-specific effect of a 'foreign' 3'UTR because addition of a different 'foreign' 3'UTR, that from albumin, to globin (SSGA; Figure 6) did not reduce the redirection by the signal sequence. The data indicate therefore that when a hybrid transcript contains both a signal sequence and a 3'UTR localisation signal there is a competition between the 5' and 3' signals. The c-myc 3'UTR localisation signal thus appears to interfere with mRNA sorting via a signal sequence.

These data have important implications for biotechnology. Firstly, they demonstrate that it is possible to create stable cell lines in which a mRNA coding for an intracellular protein is redirected to the ER. Secondly, they show that if such an mRNA contains a 3'UTR localisation signal then such a signal must be removed to achieve efficient redirection.

As indicated by the present results with the albumin 3'UTR (Figure 6), it may be most appropriate to use a vector containing a 3' UTR from the mRNA for a secreted protein, particularly one from the same gene as the signal sequence being used; in this way the desired coding region is inserted between the 5'UTR/signal sequence and 3'UTR from an mRNA for a secreted protein and the problem of signal competition is abolished.

If such modified genes are to be used for commercial purposes, ideally the signal modification should not decrease mRNA stability and translational efficiency to any major extent. The data in Figures 4 & 5 show that addition of the signal sequence to globin had no effect on mRNA stability or translational efficiency. Thus, retargeting can be accompanied by a maintenance of mRNA stability and translation. Using luciferase as a reporter we have shown that retargeting of the mRNA by the albumin signal sequence is accompanied by synthesis of an immunologically recognizable protein in the ER.

In conclusion, the present results show that it possible to retarget an mRNA to the ER whilst maintaining stability and translation.

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TABLE 1

## Oligodeoxynucleotides For PCR

|   |   |
|---|---|
| 5 | KP4-GAC AAG CTG CAC GTG GAT CCT GAG AAC TTC |
|   | KP5-AAA AGA AAA Ctc TaG aTG GCC CAA TTG TAT |
|   | KP6-GGA CTT TCC AAA ATC TCG TAA CAA CTC CGC |
|   | KP7-CTG GAC AGA TGC ACc atA TGG TCT GTT TTG |
|   | KP8-GAA GTT CTC AGG ATC CAC GTG CAG CIT GTC |

10

*Table 1.* Oligos used in cloning the various expression vectors described in the text. Letters in lowercase represent changes required to create restriction sites.

15

TABLE 2

|   |      | Polysomes       | Monosomes       |
|---|------|-----------------|-----------------|
| 5 | GG   | 56.5% $\pm$ 3.0 | 43.5% $\pm$ 3.0 |
|   | SSGG | 53.9% $\pm$ 1.7 | 46.1% $\pm$ 1.7 |
|   | GM   | 51.7% $\pm$ 0.4 | 48.3% $\pm$ 0.4 |
|   | SSGM | 51.7% $\pm$ 1.1 | 48.3% $\pm$ 1.1 |

10

*Table 2.* Distribution of ribosomes in Polysomes and Monosomes. Percentage distribution of ribosomes was calculated from the amounts of  $A_{260}$ -absorbing material present in polysomes and monosomes separated by sucrose density gradient centrifugation. Results are means  $\pm$  S.E.M. (n=6 for GG and SSGG, n=9 for GM and SSGM).

15

20

TABLE 3

|   |      | Polysomes | Monosomes |
|---|------|-----------|-----------|
| 5 | GG   | 32.3%     | 67.7%     |
|   | SSGG | 42.1%     | 57.9%     |
|   | GM   | 57.8%     | 42.2%     |
|   | SSGM | 48.5%     | 51.5%     |

10

*Table 3.* Distribution of globin transcript in polysomes and monosomes. RNA was extracted from polysome and monosomes fractions recovered from sucrose density gradients (profiles shown in Figure 5) and analysed for globin mRNA abundance by Northern Blotting.

15

CLAIMS

1. An RNA molecule encoding a mammalian signal peptide operatively linked to a protein that would normally not be secreted from a mammalian cell, said signal peptide allowing at least some of said protein to be synthesised on the endoplasmic reticulum in a manner so that it can be secreted, the molecule comprising a deletion, insertion or substitution in respect of all or part of a 3' untranslated region, relative to the corresponding region present in naturally occurring RNA encoding said protein, such that the region's effect in directing molecules to an intracellular location other than the endoplasmic reticulum or to free and/or to cytoskeletal bound polysomes is eliminated or reduced relative to the corresponding naturally occurring sequence.
2. An RNA molecule according to claim 1, wherein said signal sequence is a signal sequence selected from a growth hormone, a milk protein or albumin.
3. A DNA molecule that can be transcribed to provide an RNA molecule according to claim 1 or claim 2.
4. A nucleic acid molecule capable of hybridising to a molecule as described in any preceding claim.
5. A molecule according to any preceding claim in the form of a vector.
6. A mammalian cell comprising a molecule according to any preceding claim, when present in cell culture or in a non-human animal.

7. A method of obtaining a protein from a mammalian cell, comprising expressing the protein in the cell using a molecule according to any of claims 1 to 3 or 5 and allowing the cell to secrete the protein.
- 5 8. A chimaeric protein comprising a mammalian signal peptide linked to a protein that would normally not be secreted from a mammalian cell, said molecule being produceable by translating an RNA molecule according to claim 1 or claim 2.

**ABSTRACT**

5 Mammalian signal peptides can be used to aid in the secretion of mammalian proteins that are normally not secreted. By inactivating signals normally present in the 3' UTRs of mRNAs encoding proteins that are normally not secreted from mammalian cells, the proportion of such mRNA molecules directed to free and cytoskeletal bound polysomes can be reduced.

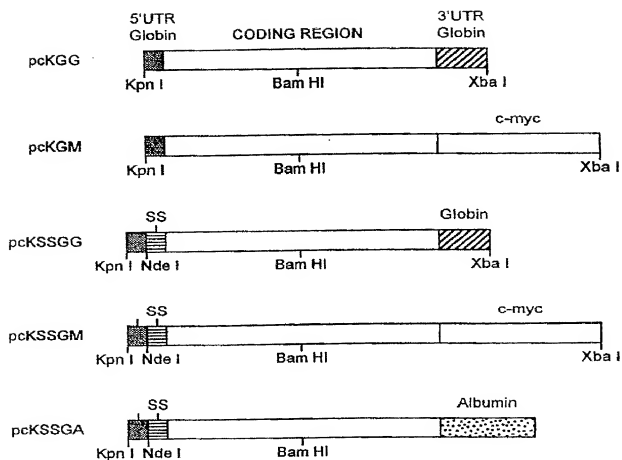


FIG. 1

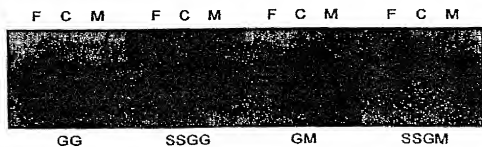


FIG. 2



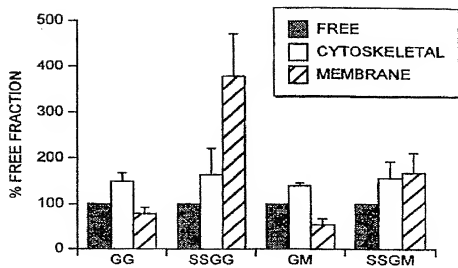


FIG. 3

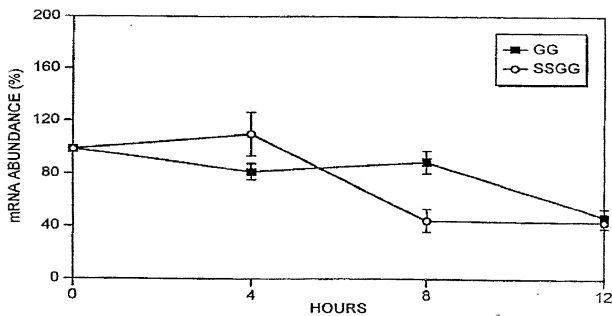


FIG. 4

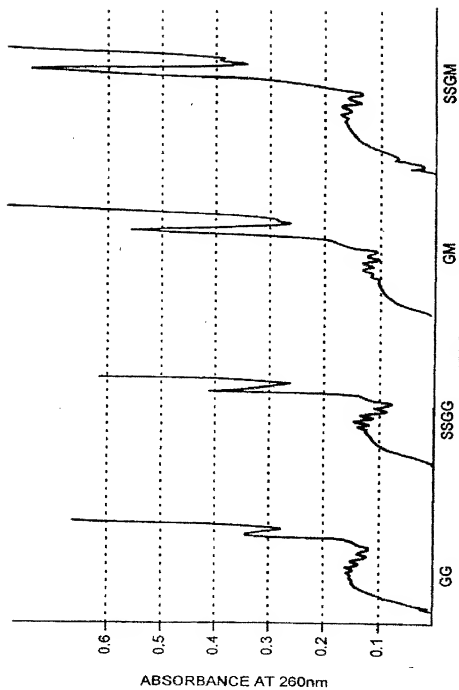


FIG. 5



FIG. 6A

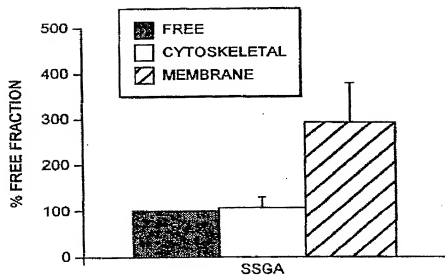


FIG. 6B